

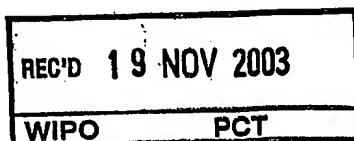


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GlaxoSmithKline Biologicals s.a.
Rue de l'Institut 89, B-1330 Rixensart, , Belgium

Patents ADP number (*if you know it*)

810 127 1001

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We request the grant of a patent on the basis of this application

Signature Michael Lubinski Date 1-Nov-02
M J Lubinski

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VACCINE

FIELD OF THE INVENTION

The present invention relates to the field of neisserial vaccine compositions, their manufacture, and the use of such compositions in medicine. More particularly it relates to processes of making novel engineered meningococcal strains which are more suitable for the production of neisserial, in particular meningococcal, outer-membrane vesicle (or bleb) vaccines. Advantageous processes and vaccine products are also described based on the use of novel LOS subunit or meningococcal outer-membrane vesicle (or bleb) vaccines which have been rendered safer for use in human subjects.

BACKGROUND OF THE INVENTION

Neisseria meningitidis (meningococcus) is a Gram negative bacterium frequently isolated from the human upper respiratory tract. It is a cause of serious invasive bacterial diseases such as bacteremia and meningitis. The incidence of meningococcal disease shows geographical, seasonal and annual differences (Schwartz, B., Moore, P.S., Broome, C.V.; Clin. Microbiol. Rev. 2 (Supplement), S18-S24, 1989). The bacterium is commonly classified according to the serogroup if its capsular polysaccharide.

Most disease in temperate countries is due to strains of serogroup B and varies in incidence from 1-10/100,000/year total population - sometimes reaching higher values (Kaczmarek, E.B. (1997), Commun. Dis. Rep. Rev. 7: R55-9, 1995; Scholten, R.J.P.M., Bijlmer, H.A., Poolman, J.T. et al. Clin. Infect. Dis. 16: 237-246, 1993; Cruz, C., Pavez, G., Aguilar, E., et al. Epidemiol. Infect. 105: 119-126, 1990).

Epidemics dominated by serogroup A meningococci, mostly in central Africa, sometimes reach incidence levels of up to 1000/100,000/year (Schwartz, B., Moore, P.S., Broome, C.V. Clin. Microbiol. Rev. 2 (Supplement), S18-S24, 1989). Nearly all cases as a whole of meningococcal disease are caused by serogroup A, B, C, W-135 and Y meningococci, and a tetravalent A, C, W-135, Y capsular polysaccharide vaccine is available (Armand, J., Arminjon, F., Mynard, M.C., Lafaix, C., J. Biol. Stand. 10: 335-339, 1982).

The frequency of *Neisseria meningitidis* infections has risen in the past few decades in many European countries. This has been attributed to increased

transmission due to an increase in social activities (for instance swimming pools, theatres, etc.). It is no longer uncommon to isolate *Neisseria meningitidis* strains that are less sensitive or resistant to some of the standard antibiotics. This phenomenon has created an unmet medical need and demand for new anti-microbial agents, vaccines, drug screening methods, and diagnostic tests for this organism.

The available polysaccharide vaccines are currently being improved by way of chemically conjugating them to carrier proteins (Lieberman, J.M., Chiu, S.S., Wong, V.K., et al. JAMA 275 : 1499-1503, 1996).

A serogroup B vaccine, however, is not available. The serogroup B capsular polysaccharide has been found to be nonimmunogenic - most likely because it shares structural similarity with host components (Wyle, F.A., Artenstein, M.S., Brandt, M.L. et al. J. Infect. Dis. 126: 514-522, 1972; Finne, J.M., Leinonen, M., Mäkelä, P.M. Lancet ii.: 355-357, 1983). Effort has therefore been focused in trying to develop serogroup B vaccines from outer membrane vesicles (or blebs) or purified protein components therefrom.

Alternative meningococcal antigens for vaccine development are meningococcal lipooligosaccharides (LOS). These are outer membrane bound glycolipids which differ from the lipopolysaccharides (LPS) of the Enterobacteriaceae by lacking the O side chains, and thus resemble the rough form of LPS (Griffiss et al. Rev Infect Dis 1988; 10: S287-295). Heterogeneity within the oligosaccharide moiety of the LOS generates structural and antigenic diversity among different meningococcal strains (Griffiss et al. Inf. Immun. 1987; 55: 1792-1800). This has been used to subdivide the strains into 12 immunotypes. Immunotypes L3, L7, L9 have an identical carbohydrate structure and have therefore been designated L3,7,9 (or, for the purposes of this specification, generically as "L3"). Meningococcal LOS L3,7,9 (L3), L2 and L5 can be modified by sialylation, or by the addition of cytidine 5'-monophosphate-N-acetylneuraminic acid. Antibodies to LOS have been shown to protect in experimental rats against infection and to contribute to the bactericidal activity in children infected with *N. meningitidis* (Griffiss et al J Infect Dis 1984; 150: 71-79).

A problem associated with the use of LOS in a meningococcal vaccine, however, is its toxicity (due to its Lipid A moiety).

LOS is also present on the surface of meningococcal blebs. For many years efforts have been focused on developing meningococcal outer membrane vesicle (or

bleb) based vaccines (de Moraes, J.C., Perkins, B., Camargo, M.C. et al. Lancet 340: 1074-1078, 1992; Bjune, G., Hoiby, E.A. Gronnesby, J.K. et al. 338: 1093-1096, 1991). Such vaccines have the advantage of including several integral outer-membrane proteins in a properly folded conformation which can elicit a protective immunological response when administered to a host. In addition, Neisserial strains (including *N. meningitidis* serogroup B - menB) excrete outer membrane blebs in sufficient quantities to allow their manufacture on an industrial scale. More often, however, blebs are prepared by methods comprising a detergent (e.g. deoxycholate) extraction of the bacterial cells (e.g. EP 11243), which has the effect of removing a lot of the LOS from the vaccine. This is desired due to the toxicity of LOS (also called endotoxin) as described above.

A further problem with using LOS as a vaccine antigen is that 12 LPS immunotypes exist with a diverse range of carbohydrate-structures (M. P. Jennings *et al*, Microbiology 1999, 145, 3013-3021). Antibodies raised against one immunotype fail to recognise a different immunotype. Although effort has been focused on producing a generic "core" region of the oligosaccharide portions of the LOS immunotypes (e.g. WO 94/08021), the bactericidal activity of antibodies generated against the modified LOS is lost. Thus a vaccine may need to have many LOS components of different immunotype to be effective.

A further problem exists with the use of LOS (also known as LPS or lipopolysaccharide) as antigens in human vaccines, namely that they carry saccharide structures that are similar to human saccharide structures, thus posing a safety issue with their use.

The present invention presents processes for ameliorating one or more of the above problems, and presents methods for making novel vaccines based on meningococcal LOS as a protective antigen, particularly when present on an outer membrane vesicle.

DESCRIPTION OF THE INVENTION

The subject matter of and information disclosed within the publications and patents or patent applications mentioned in this specification are incorporated by reference herein.

Reference to "lipooligosaccharide" (or "LOS") may also be referred to as "lipopolysaccharide" or "LPS".

The present inventors have found that the shortening LOS oligosaccharide structures leads to the loss of epitopes that can elicit a bacteriocidal immune response.

5 Instead, the inventors have found that in order to use LOS most effectively in a vaccine formulation, the LOS oligosaccharide structure must be retained as much as possible, but a combination of just 2 LOS antigens can yield a universally effective Neisserial (preferably meningococcal) vaccine. A first aspect of the invention is an immunogenic composition for the prevention or treatment of Neisserial (preferably
10 meningococcal or meningococcal B) disease comprising Neisserial (preferably meningococcal) LOS of immunotype L2 and LOS of immunotype L3. LOS may be isolated by either known purification procedures, or may be present in at least 2 outer membrane vesicle (or bleb) preparations derived from L2 and L3 Neisserial strains. In order to remove toxic loosely held LOS from the bleb preparation, but retain high
15 levels of integrated LOS antigen in the bleb, it is preferred that the blebs are extracted using a low concentration of detergent - 0.0.3%, preferably 0.05-2%, most preferably around 0.1%, preferably deoxycholate (or DOC). Such a combination of LOS antigens is surprisingly advantageous in being effective against over 90% of *N meningitidis* strains.

20 The inventors have also found that bleb the above bleb immunogenic compositions of the invention, and indeed any Neisserial (preferably gonococcal or meningococcal) derived bleb immunogenic composition, can have enhanced effect of protective antigens (including LOS) on their surface if certain combinations of immunodominant outer membrane proteins are downregulated in expression (and
25 preferably deleted). A second aspect of the invention is therefore a Neisserial bleb preparation derived from a neisserial strain which has had 2 or more of the following outer membrane proteins downregulated in expression, and preferably deleted, compared to the native, non-modified strain: PorA, PorB, OpA, OpC or PilC. Preferably PorA and OpA, PorA and OpC, OpA and OpC, or PorA & OpA & and
30 OpC are downregulated or deleted. Such mutations are beneficial in any Neisserial (preferably meningococcal) strain from which bleb immunogenic compositions are to be derived, however it is preferred that L2 or L3 immuntype Neisserial (preferably meningococcal) strains are used. Preferably the bleb immunogenic compositions of

the invention contains both L2 and L3 blebs where at least one (and preferably both) is deficient in the above combinations of immunodominant outer membrane proteins (or OMPs).

5 Safety of the above LPS immunogenic compositions

The safety of antibodies raised to L3 or L2 LPS has been questioned, due to the presence of a structure similar to the lacto-N-neotetraose oligosaccharide group present in human glycosphingolipids. Even if a large number of people has been safely vaccinated with deoxycholate extracted vesicle vaccines containing residual amount
10 of L3 LPS (G. Bjune *et al*, Lancet (1991), 338, 1093-1096; GVG. Sierra *et al*, NIPH ann (1991), 14, 195-210), the deletion of the terminal part of the LOS saccharidic is advantageous in preventing any cross-reaction with structures present at the surface of human tissues. Inactivation of the *lgtB* gene results in an intermediate LPS structure in which the terminal galactose residue and the sialic acid are absent (see figure 1 and 2).
15 Such intermediates could be obtained in an L3 and an L2 LPS strain. An alternative and less preferred (short) version of the LPS can be obtained by turning off the *lgtE* gene. LgtB mutants are deleted as the inventors have found that the safety issue can be resolved whilst still retaining the LPS protective oligosaccharide epitopes.

Therefore, the above L2 or L3 preparations (whether purified or in an isolated
20 bleb) or meningococcal bleb preparations in general advantageously are derived from a Neisserial strain (preferably meningococcal) that has been genetic engineered to permanently downregulate the expression of functional gene product from the *lgtB* or *lgtE* gene, preferably by switching the gene off, most preferably by deleting all or part of the promoter and/or open-reading frame of the gene.

25 Where the above bleb preparations are derived from a meningococcus B strain, it is further preferred that the capsular polysaccharide (which also contains human-like saccharide structures) is also removed. Although many genes could be switched off to achieve this, the inventors have advantageously shown that it is preferred that the bleb production strain has been genetically engineered to permanently downregulate the
30 expression of functional gene product from the *siaD* gene (i.e. downregulating α -2-8 polysialyltransferase activity), preferably by switching the gene off, most preferably by deleting all or part of the promoter and/or open-reading frame of the gene. Such an inactivation is described in WO 01/09350. The *siaD* (also known as *synD*) mutation is

the most advantageous of many mutations that can result in removing the human-similar epitope from the capsular polysaccharide, because it one of the only mutations that has no effect on the biosynthesis of the protective epitopes of LOS, thus being advantageous in a process which aims at ultimately using LOS as a protective antigen, and has a minimal effect on the growth of the bacterium. A preferred aspect of the invention is therefore a bleb immunogenic preparation as described above which is derived from an *lgtE*⁻ *siaD*⁻ and preferably an *lgtB*⁻ *siaD*⁻ meningococcus B mutant strain. The strain itself is a further aspect of the invention.

Although *siaD*⁻ mutation is preferable for the above reasons, other mutations which switch off meningococcus B capsular polysaccharide synthesis may be used. Thus bleb production strain can be genetically engineered to permanently downregulate the expression of functional gene product from one or more of the following genes: *ctrA*, *ctrB*, *ctrC*, *ctrD*, *synA* (equivalent to *synX* and *siaA*), *synB* (equivalent to *siaB*) or *synC* (equivalent to *siaC*) genes, preferably by switching the gene off, most preferably by deleting all or part of the promoter and/or open-reading frame of the gene. The *lgtE*⁻ mutation may be combined with one or more of these mutations. Preferably the *lgtB*⁻ mutation is combined with one or more of these mutations. A further aspect of the invention is therefore a bleb immunogenic preparation as described above which is derived from such a combined mutant strain of meningococcus B. The strain itself is a further aspect of the invention.

A Neisserial locus containing various *lgt* genes, including *lgtB* and *lgtE*, and its sequence is known in the art (see M. P. Jennings *et al*, Microbiology 1999, 145, 3013-3021 and references cited therein).

The Toxicity of LOS

The above purified LOS or bleb immunogenic compositions of the invention may also be rendered less toxic by downregulating expression of certain genes in the bacterial production strain from which they are derived. Although such detoxification may not necessary for intranasal immunization with native OMV (J.J. Drabick *et al*, Vaccine (2000), 18, 160-172), for parenteral vaccination detoxification would present an advantage. Preferably the LOS purified LOS or bleb immunogenic compositions of the invention are detoxified by genetically engineering the Neisserial production strain by mutation/modification/inactivation of the genes involved in LipidA biosynthesis,

particularly those genes involved in adding secondary acyl chains to lipidA, in particular by downregulating the expression of functional gene product from the msbB and/or htrB genes, and preferably by switching the gene off, most preferably by deleting all or part of the promoter and/or open-reading frame of the gene.

- 5 Alternatively (or in addition) the purified LOS or bleb immunogenic compositions can be derived from a Neisserial strain which has been genetically modified so that one or more of the following genes are upregulated (by introducing a stronger promoter or integrating an extra copy of the gene): pmrA, pmrB, pmrE and pmrF. See WO 01/09350 for more detail on the above detoxification methods, and for relevant promoter / gene sequences and upregulation and downregulation methods.

- 10 For instance, by "downregulating the expression of functional gene product" it is meant that additions, deletions or additions are made to the promoter or open reading frame of the gene in question such that the biosynthetic activity of the total gene product reduces (by 60, 70, 80, 90, 95 or most preferably 100%). Clearly frameshift mutations may be introduced, or weaker promoters substituted, however
15 most preferably most or all of the open reading frame and/or promoter is deleted to ensure a permanent downregulation of the gene product (as described in WO 01/09350).

- 20 Further aspects of the invention include the above described genetically modified Neisserial (preferably meningococcal or gonococcal or meningococcal B) strains from which the LOS or bleb immunogenic preparations of the invention may be derived.

25 The LOS or LOS-containing bleb preparations of the invention

- A further aspect of the invention are LOS preparations (particularly those described above) isolated from the Neisserial strains of the invention. Preferably the isolated LOS (or LOS-containing bleb) is L2 or L3 immunotype, and preferably the
30 immunogenic compositions of the invention comprise both L2 and L3 LOS (or bleb) preparations of the invention.

Such preparations may also be improved by conjugating the above LOS (whether purified or present in a bleb preparation) to a carrier comprising a source of

T-cell epitopes (rendering the LOS an even better immunogen). A purified LOS preparation of the invention may alternatively (or in addition) be rendered a better antigen by presenting it in liposome formulations known in the art.

5 The process of isolation of LOS from bacteria is well known in the art (see for instance the hot water-phenol procedure of Wesphal & Jann [Meth. Carbo. Chem. 1965, 5:83-91]).

10 For the purposes of this invention "a carrier comprising a source of T-cell epitopes" is usually a peptide or, preferably, a polypeptide or protein. Conjugation techniques are well known in the art. Typical carriers include protein D from non typeable *H. influenzae*, tetanus toxoid, diphtheria toxoid, and CRM197.

15 Where the LOS of the invention is present in a bleb formulation the LOS is preferably conjugated in situ by methods allowing the conjugation of LOS to one or more outer membrane proteins also present on the bleb preparation (e.g. PorA or PorB) (e. g. by the conjugation chemistry described in Devi *et al*, Infect Immun (1997), 65, 1045-1052, or other treatments capable to create covalent links between molecules). This process can advantageously enhance the stability and/or immunogenicity (providing T-cell help) and/or antigenicity of the LOS antigen within the bleb formulation. One or more of Men A, C, Y or W capsular polysaccharides or oligosaccharides (preferably at least MenC, MenA and MenC, or Men C and MenY)
20 may also be conjugated onto an outermembrane protein of the bleb of the invention as well.

Outer Membrane Vesicles (OMVs or blebs) can be isolated by many known techniques (Fredriksen *et al*, NIPH Annals (1991), 14, 67-79; Zollinger *et al*, J. Clin Invest (1979), 63, 836-848; Saunders *et al*, Infect Immun (1999), 67, 113-119; J.J. Drabick *et al*, Vaccine (1999), 18, 160-172). These divide into 2 main groups – techniques which use deoxycholate (about 0.5%) to extract blebs from meningococcus, and techniques that use low levels of deoxycholate (DOC) or no deoxycholate at all. DOC free process blebs have the interesting feature of
30 maintaining high level of LOS in the OMV – which is advantageous in a vaccine where LOS is a protective antigen. Compared to DOC extracted blebs, the concentration of L3 Ags in OMV obtained by a DOC free process is approximately ten times higher, also taking into account the fixing of *lgtA*. A detergent-free

(preferably DOC-free) process of preparing blebs is preferred for the purposes of the processes of this invention for this reason, although extraction with a buffer containing low levels of detergent (preferably DOC) may also be advantageous in that the step would leave most of the tightly interacting LOS in the bleb whilst removing any more toxic loosely retained LOS. Preferably 0-0.3% detergent (preferably DOC) is used for bleb extraction, more preferably 0.05-0.2%, and most preferably around 0.1%. DOC free (or low DOC) extraction processes are particularly preferred where the LOS has been detoxified by one of the methods detailed above.

The immunogenic compositions of the invention may readily be formulated as vaccine compositions using a pharmaceutically acceptable excipient.

A process for making the Neisserial (preferably meningococcal) immunogenic compositions or vaccines of the invention is further provided comprising the steps of isolating, purified LOS of the invention (preferably L2 or L3) as described above or producing isolated blebs of the invention (preferably with an L2 or L3 immunotype) as described above, and formulating the LOS or blebs with a pharmaceutically acceptable excipient. Preferably purified LOS of both immunotype L2 and L3 of the invention, or blebs of both immunotype L2 and L3 of the invention, or a purified LOS of L2 and a bleb of L3 (or vice versa), are combined in a mixing step. Preferably the purified LOS or bleb of the invention has been conjugated as described above after isolation. An additional liposome formulation step may also be added for the purified LOS. Preferably bleb preparations are isolated by extraction with low (or no) concentrations of DOC (as described above).

Such L2 and L3 combination processes can yield a vaccine which is effective against almost all meningococcal B strains.

The above immunogenic compositions (or processes) may have added one or more (2, 3 or 4) meningococcal polysaccharides (either plain or conjugated to a carrier comprising T-cell epitopes) from serogroups A, C, Y or W to the composition. Preferably at least C is added (most preferably conjugated), and more preferably A and C or Y and C (preferably all conjugated) and most preferably A, C, Y and W (preferably all conjugated). The term "polysaccharide" is intended to cover unsized or sized (reduced in size) polysaccharides, or sized oligosaccharides. Advantageously a

conjugated H. influenzae B capsular polysaccharide is also included in the above compositions to generate a universal meningitis vaccine.

Vaccine Formulations of the invention

5 The immunogenic compositions of the invention may be formulated with a suitable adjuvant to generate vaccine compositions of the invention.

Suitable adjuvants include an aluminium salt such as aluminum hydroxide gel (alum) or aluminium phosphate (preferably aluminium hydroxide), but may also be a salt of calcium (particularly calcium carbonate), iron or zinc, or may be an insoluble
10 suspension of acylated tyrosine, or acylated sugars, cationically or anionically derivatised polysaccharides, or polyphosphazenes.

Suitable Th1 adjuvant systems that may be added include, Monophosphoryl lipid A, particularly 3-de-O-acylated monophosphoryl lipid A (or other non-toxic derivatives of LPS), and a combination of monophosphoryl lipid A, preferably 3-de-
15 O-acylated monophosphoryl lipid A (3D-MPL) [or non toxic LPS derivatives] together with an aluminium salt. An enhanced system involves the combination of a monophosphoryl lipid A and a saponin derivative particularly the combination of QS21 [or other saponin] and 3D-MPL [or non toxic LPS derivative] as disclosed in WO 94/00153, or a less reactogenic composition where the QS21 [or saponin] is
20 quenched with cholesterol as disclosed in WO96/33739. A particularly potent adjuvant formulation involving QS21, 3D-MPL and tocopherol in an oil in water emulsion is described in WO95/17210 and is a preferred formulation that may be added. Other adjuvants that may be added comprise a saponin, more preferably QS21 and/or an oil in water emulsion and tocopherol. Unmethylated CpG containing oligo
25 nucleotides (WO 96/02555) may also be added

Vaccine preparation is generally described in Vaccine Design ("The subunit and adjuvant approach" (eds Powell M.F. & Newman M.J.) (1995) Plenum Press New York).

An immunoprotective dose of vaccines can be administered via the systemic or
30 mucosal route. These administrations may include injection *via* the intramuscular, intraperitoneal, intradermal or subcutaneous routes; or *via* mucosal administration to the oral/alimentary, respiratory, genitourinary tracts. Typically bleb quantity in each vaccine dose is selected as an amount which induces an immunoprotective response

without significant, adverse side effects in typical vaccinees. Such amount will vary depending upon which specific immunogen is employed and how it is presented. Generally, it is expected that each dose will comprise 1-100µg of each bleb, preferably 5-50µg, and most typically in the range 5 - 25µg.

5

Further improvements to the bleb immunogenic compositions of the invention

The above bleb compositions of the invention may be further improved in efficacy in vaccines of the invention if the Neisserial strain from which they are derived (including gonococcus, and preferably meningococcus, most preferably *N. meningitidis* B) have one or more of the following genes (encoding protective antigens) upregulated by inserting further copies of the gene into the genome, or introducing a stronger promoter upstream of the existing gene, or any of the other ways discussed in WO 01/09350 which are capable of inducing modified strains to make over 1.2, 1.5, 2, 3, 5 or 10 times the level of antigen as compared to the unmodified strain: NspA (WO 96/29412), Hsf-like (WO 99/31132), Hap (PCT/EP99/02766), OMP85 (WO 00/23595), PilQ (PCT/EP99/03603), PldA (PCT/EP99/06718), FrpB (WO 96/31618), TbpA (WO92/03467, US5912336, WO93/06861 and EP586266), TbpB (WO93/06861 and EP586266), NadA (Comanducci et al J. Exp. Med. 2002 195; 1445-1454), FrpA/FrpC or portions in common between these antigens involving 5 or more repeat sequences (WO 92/01460; Thompson *et al.*, (1993) J. Bacteriol. 175:811-818; Thompson *et al.*, (1993) Infect. Immun.. 61:2906-2911), LbpA, LbpB (PCT/EP98/05117), FhaB (WO98/02547 SEQ ID NO 38 [nucleotides 3083-9025]), HasR (PCT/EP99/05989), lipo02 (PCT/EP99/08315), Tbp2 (WO 99/57280), MltA (WO 99/57280), TspA (WO 00/03003), TspB (WO 00/03003) and ctrA (PCT/EP00/00135). It is particularly preferred if both Hsf and TbpA (Low or High, or both Low and High molecular weight forms [EP 586266]), or Hsf and OMP85, or OMP85 and TbpA (Low or High, or both Low and High molecular weight forms), or NspA and Hsf, or NspA and OMP85, or NspA and TbpA (Low or High, or both Low and High molecular weight forms) are both upregulated.

Ghost or Killed Whole cell vaccines

The inventors envisage that the above compositions and vaccines concerning blebs can be easily extended to processes concerning ghost or killed whole cell preparations and vaccines (with identical advantages). Methods of making ghost preparations (empty cells with intact envelopes) from Gram-negative strains are well known in the art (see for example WO 92/01791). Methods of killing whole cells to make inactivated cell preparations for use in vaccines are also well known. Therefore the compositions and vaccines involving blebs described throughout this document are envisioned to be applicable to the the same compositions or vaccines comprising equivalent ghost and killed whole cell preparations of the invention.

Serum bactericidal assay

The serum bactericidal assay is the preferred method to assess synergistic relationships between antigens when combined in an immunogenic composition of the invention.

Such a synergistic response may be characterised by the SBA elicited by the combination of antigens being at least 50%, two times, three times, preferably four times, five times, six times, seven times, eight times, nine times and most preferably ten times higher than the SBA elicited by each antigen separately. Preferably SBA is measured against a homologous strain from which the antigens are derived and preferably also against a panel of heterologous strains. (See below for a representative panel for instance BZ10 (B:2b:P1.2) belonging to the A-4 cluster; B16B6 (B:2a:P1.2) belonging to the ET-37 complex; and H44/76 (B:15:P1.7,16)). SBA is the most commonly agreed immunological marker to estimate the efficacy of a meningococcal vaccine (Perkins et al. J Infect Dis. 1998, 177:683-691). Satisfactory SBA can be ascertained by any known method. SBA can be carried out using sera obtained from animal models, or from human subjects.

A preferred method of conducting SBA with human sera is the following. A blood sample is taken prior to the first vaccination, two months after the second vaccination and one month after the third vaccination (three vaccinations in one year

being a typical human primary vaccination schedule administered at, for instance, 0, 2 and 4 months, or 0, 1 and 6 months). Such human primary vaccination schedules can be carried out on infants under 1 year old (for instance at the same time as Hib vaccinations are carried out) or 2-4 year olds or adolescents may also be vaccinated to
5 test SBA with such a primary vaccination schedule. A further blood sample may be taken 6 to 12 months after primary vaccination and one month after a booster dose, if applicable.

SBA will be satisfactory for an antigen or bleb preparation with homologous bactericidal activity if one month after the third vaccine dose (of the primary
10 vaccination schedule) (in 2-4 year olds or adolescents, but preferably in infants in the first year of life) the percentage of subjects with a four-fold increase in terms of SBA (antibody dilution) titre (compared with pre-vaccination titre) against the strain of meningococcus from which the antigens of the invention were derived is greater than 30%, preferably greater than 40%, more preferably greater than 50%, and most
15 preferably greater than 60% of the subjects.

Of course an antigen or bleb preparation with heterologous bactericidal activity can also constitute bleb preparation with homologous bactericidal activity if it can also elicit satisfactory SBA against the meningococcal strain from which it is derived.

20 SBA will be satisfactory for an antigen or bleb preparation with heterologous bactericidal activity if one month after the third vaccine dose (of the primary vaccination schedule) (in 2-4 year olds or adolescents, but preferably in infants in the first year of life) the percentage of subjects with a four-fold increase in terms of SBA (antibody dilution) titre (compared with pre-vaccination titre) against three
25 heterologous strains of meningococcus is greater than 20%, preferably greater than 30%, more preferably greater than 35%, and most preferably greater than 40% of the subjects. Such a test is a good indication of whether the antigen or bleb preparation with heterologous bactericidal activity can induce cross-bactericidal antibodies against various meningococcal strains. The three heterologous strains should preferably have
30 different electrophoretic type (ET)-complex or multilocus sequence typing (MLST) pattern (see Maiden et al. PNAS USA 1998, 95:3140-5) to each other and preferably to the strain from which the antigen or bleb preparation with heterologous bactericidal activity is made or derived. A skilled person will readily be able to determine three

strains with different ET-complex which reflect the genetic diversity observed amongst meningococci, particularly amongst meningococcus type B strains that are recognised as being the cause of significant disease burden and/or that represent recognised MenB hyper-virulent lineages (see Maiden et al. *supra*). For instance three strains that could be used are the following: BZ10 (B:2b:P1.2) belonging to the A-4 cluster; B16B6 (B:2a:P1.2) belonging to the ET-37 complex; and H44/76 (B:15:P1.7,16) belonging to the ET-5 complex, or any other strains belonging to the same ET/Cluster. Such strains may be used for testing an antigen or bleb preparation with heterologous bactericidal activity made or derived from, for instance, meningococcal strain CU385 (B:4:P1.15) which belongs to the ET-5 complex. Another sample strain that could be used is from the Lineage 3 epidemic clone (e.g. NZ124 [B:4:P1.7,4]). Another ET-37 strain is NGP165 (B:2a:P1.2).

Processes for measuring SBA activity are known in the art. For instance a method that might be used is described in WO 99/09176 in Example 10C. In general terms, a culture of the strain to be tested is grown (preferably in conditions of iron depletion – by addition of an iron chelator such as EDDA to the growth medium) in the log phase of growth. This can be suspended in a medium with BSA (such as Hanks medium with 0.3% BSA) in order to obtain a working cell suspension adjusted to approximately 20000 CFU/ml. A series of reaction mixes can be made mixing a series of two-fold dilutions of sera to be tested (preferably heat-inactivated at 56°C for 30 min) [for example in a 50µl/well volume] and the 20000 CFU/ml meningococcal strain suspension to be tested [for example in a 25µl/well volume]. The reaction vials should be incubated (e.g. 37°C for 15 minutes) and shaken (e.g. at 210 rpm). The final reaction mixture [for example in a 100µl volume] additionally contains a complement source [such as 25 % final volume of pretested baby rabbit serum], and is incubated as above [e.g. 37°C for 60 min]. A sterile polystyrene U-bottom 96-well microtiter plate can be used for this assay. A aliquot [e.g. 10 µl] can be taken from each well using a multichannel pipette, and dropped onto Mueller-Hinton agar plates (preferably containing 1 % Isovitalex and 1 % heat-inactivated Horse Serum) and incubated (for example for 18 hours at 37°C in 5 % CO₂). Preferably, individual colonies can be counted up to 80 CFU per aliquot. The following three test samples can be used as controls: buffer + bacteria + complement; buffer + bacteria + inactivated complement; serum + bacteria + inactivated complement. SBA titers can be straightforwardly

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calculated using a program which processes the data to give a measurement of the dilution which corresponds to 50 % of cell killing by a regression calculation.

All references or patent applications cited within this patent specification are
5 incorporated by reference herein.

EXAMPLES

The examples below are carried out using standard techniques, which are well known and routine to those of skill in the art, except where otherwise described in detail.

5 The examples are illustrative, but do not limit the invention.

Example 1:

Examples describing deletions genes encoding proteins involved in B capsular polysaccharide production of meningococcus B, the deletion of the PorA gene, the upregulation of various protective outer membrane proteins on the surface of meningococcal blebs, the downregulation of immunodominant proteins or biosynthetic enzymes, and processes for isolating blebs are described in WO 01/09350.

15 **Example 2:** Experiments with L3 and intermediate (*lgtB*⁻) DOC free blebs (non-detoxified LOS) induced cross-bactericidal antibodies

The MC58 meningococcal derivative strain used is B:P1.7.16, *opc*⁻, *siaD*⁻. This strain was genetically modified to express either L3 (strain 2G2) or an intermediate epitope (strain 2G EcoN1b-1), as 2G2 but *lgtB* additionally turned off) or an LPS in short version (strain C6, *lgtE* off). OMV were produced according either a normal high DOC process or DOC free process.

Mice (10 per group) were immunized three times by the intra-muscular route on Day 0, 20 and 28. They received 1 or 10 µg (protein content) of blebs formulated on Al(OH)₃. Blood samples were taken on day 28 (post II) and day 42 (post III).

Bactericidal assays were done on pooled sera and using homologous strains (MC58 and H44/76) and two heterologous strains (M97250687 and M9725078) with baby rabbit serum as source of exogenous complement.

The following table summarizes the results (bactericidal titers for 50% killing):

Antigen	Blood samples	Strain and serotype			
		MC58 P1.7.16	H44/76TT P1.7.16	M97250687 P1.19.15	M97252078 P1.4
c6 no doc 10ug IM	Post II	>2560	>2560	>2560	98
c6 no doc 10ug IM	Post III	1 353	>2560	>2560	90
c6 no doc 1ug IM	Post II	247	620	247	<20
c6 no doc 1ug IM	Post III	411	878	748	<20
2g2 no doc 10ug IM	Post II	>320	>2560	>2560	>2560
2g2 no doc 10ug IM	Post III	>2560	>2560	>2560	1407
2g2 no doc 1ug IM	Post II	>2560	>2560	>2560	119
2g2 no doc 1ug IM	Post III	>2560	>2560	>2560	348
2gecoN1b-1 no doc 10ug IM	Post II	>2560	>2560	>2560	1162
2gecoN1b-1 no doc 10ug IM	Post III	>2560	>2560	>2560	1213
2gecoN1b-1 no doc 1ug IM	Post II	1 151	>2560	1 696	22
2gecoN1b-1 no doc 1ug IM	Post III	2 220	>2560	1 947	135
c6 doc 10ug IM	Post II	308	248	341	<20
c6 doc 10ug IM	Post III	189	104	400	<20
c6 doc 1ug IM	Post II	33	43	63	<20
c6 doc 1ug IM	Post III	NC (>20)	24	156	<20
2g2 doc 10ug IM	Post II	NC (>20)	25	360	<20
2g2 doc 10ug IM	Post III	201	<20	647	<20
2g2 doc 1ug IM	Post II	275	<20	299/644	<20
2g2 doc 1ug IM	Post III	237	<20	728	<20
2gecoN1b-1 doc 10ug IM	Post II	573	31	685	<20
2gecoN1b-1 doc 10ug IM	Post III	NC (>40)	21	1 140	<20
2gecoN1b-1 doc 1ug IM	Post II	261	NC	118	<20
2gecoN1b-1 doc 1ug IM	Post III	348	NC	692	<20

Clearly, the presence of L3 (2g2) or intermediate (2gecon1b-1) epitope induces cross-bactericidal antibodies, while blebs from truncated LPS strain (C6) induce lower level of cross-reacting antibodies. This was particularly illustrated when 1µg of OMV was injected.

Moreover, as shown with OMV purified with DOC, reducing the LPS content of blebs reduces the induction of cross-bactericidal antibodies. Aside from increased LPS, it is possible that DOC free blebs may also advantageously retain some proteins loosely interacting with the OMVs such as lipoproteins.

We Claim:

1. A Neisserial bleb preparation derived from a neisserial strain which has had 2
5 or more of the following outer membrane proteins downregulated in expression, and preferably deleted, compared to the native strain: PorA, PorB, OpA, OpC or PilC.
2. A Neisserial bleb preparation derived from a neisserial strain with an L2 LOS
10 immunotype which has had 1 or more of the following outer membrane proteins downregulated in expression, and preferably deleted, compared to the native strain: PorA, PorB, OpA, OpC or PilC.
3. A Neisserial bleb preparation derived from a neisserial strain with an L3 LOS
15 immunotype which has had 1 or more of the following outer membrane proteins downregulated in expression, and preferably deleted, compared to the native strain: PorA, PorB, OpA, OpC or PilC.
4. A multivalent Neisserial bleb preparation derived from a neisserial strain with
20 an L2 LOS immunotype and a neisserial strain with an L3 LOS immunotype, wherein one or more of said strains has had 1 or more of the following outer membrane proteins downregulated in expression, and preferably deleted, compared to the native strain: PorA, PorB, OpA, OpC or PilC.
5. A meningococcal serogroup B bleb preparation derived from a meningococcal
25 serogroup B strain which has had either or both of the following genes downregulated in expression, and preferably deleted, compared to the native strain: siaD [or, alternatively, ctrA, ctrB, ctrC, ctrD, synA (equivalent to synX and siaA), synB (equivalent to siaB) or synC (equivalent to siaC)] and lgtB.
- 30 6. The Neisserial bleb preparation of claims 1-3 or the multivalent Neisserial bleb preparation of claim 4 derived from meningococcal serogroup B strains as claimed in claim 5.

7. The Neisserial bleb preparation or the multivalent Neisserial bleb preparation of any one of claims 1-6 derived from neisserial strains which have had any of the following combinations of outer membrane proteins downregulated in expression, and preferably deleted, compared to the native strains: PorA and OpA, PorA and OpC,
5 OpA and OpC, PorA and OpA and OpC.
8. The Neisserial bleb preparation or the multivalent Neisserial bleb preparation of any one of claims 1-7 derived from detoxified neisserial strains which have had either or both of the following combinations of genes downregulated in expression,
10 and preferably deleted, compared to the native strains: msbB or htrB.
9. The Neisserial strain from which the Neisserial bleb preparations of claims 1-8 are derived.
- 15 10. A LOS preparation isolated from the Neisserial strain of claim 9.
11. The LOS preparation of claim 10 comprising immunotype L2 and L3 LOS.
12. The LOS preparation of claim 10 or 11 in a liposome formulation.
20
13. The Neisserial bleb preparation of any one of claims 1-8 or the LOS preparation of claims 10-12, wherein the LOS contained therein is conjugated to a source of T-helper epitopes, preferably a protein or outer membrane protein.
- 25 14. An immunogenic composition or vaccine comprising the Neisserial bleb preparation or the LOS preparation of any one of claims 1-8 or 10-13, and a pharmaceutically acceptable excipient.
15. The vaccine of claim 14, additionally comprising an adjuvant, preferably
30 aluminium hydroxide.
16. The vaccine of claim 14 or 15 additionally comprising one or more conjugated capsular polysaccharides or oligosaccharides derived from the following strains:

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meningococcus serogroup A, meningococcus serogroup C, meningococcus serogroup W-135, meningococcus serogroup Y, and *H. influenzae* type b.

17. A process of manufacturing the Neisserial bleb preparation vaccine of claim
5 14 comprising the steps of culturing a Neisserial strain of claim 9, isolating blebs therefrom, and formulating the blebs with a pharmaceutically acceptable excipient.

18. The process of claim 17, wherein the isolation step is carried out by extracting with 0-0.3% deoxycholate.

10

ABSTRACT

The present invention relates to the field of neisserial vaccine compositions, their manufacture, and the use of such compositions in medicine. More particularly it relates to processes of making novel engineered meningococcal strains which are more suitable for the production of neisserial, in particular meningococcal, outer-membrane vesicle (or bleb) vaccines. Advantageous processes and vaccine products are also described based on the use of novel LOS subunit or meningococcal outer-membrane vesicle (or bleb) vaccines which have been rendered safer for use in human subjects. In particular combinations of gene downregulations are described such as PorA & OpA, PorA and OpC, OpA and OpC, and PorA and OpA and OpC. Additionally a combination of gene downregulations are described to render meningococcal B blebs safer: namely SiaD and lgtB.

15

Figure 1: L3 and L2 immunotypes (H44/76 , MC58 strains)

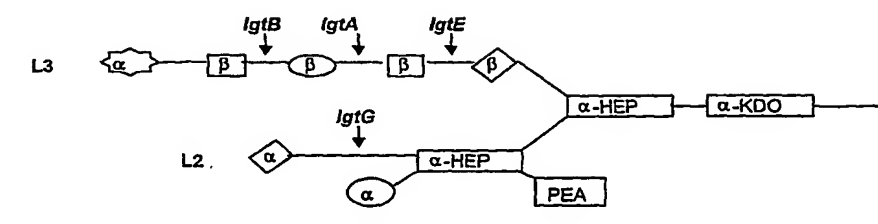
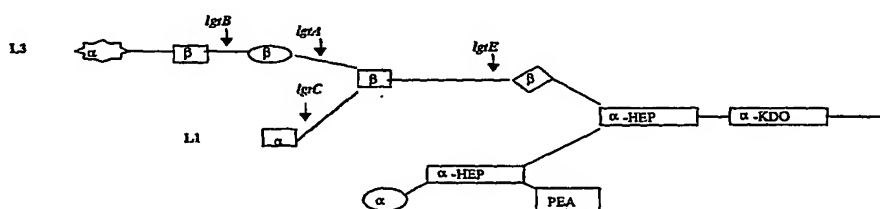







Figure 2: L3 and L1 immunotypes (e.g. 126E strain)



 Sialic acid
 glucose
 galactose

 acetylglucosamine
 phosphoethanolamine

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